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Global HIV Vaccine Research Cryorepository-GHRC

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Abstract

The Global HIV Vaccine Research Cryorepository (GHRC) is an international consortium coordinated by the Fraunhofer Institute for Biomedical Engineering (Fraunhofer IBMT) and funded by the Bill & Melinda Gates Foundation. It is one of the Centralized Service Facilities of the Collaboration for AIDS Vaccine Discovery (CAVD) whose goal is to discover and design a safe and effective preventive vaccine against HIV/AIDS by means of collaborative and comparative research, supported by the utilization of innovative concepts and new technologies.

In this context, GHRC has established the first large-scale centralized biobank for low-temperature storage of HIV-1 related specimens and reagents where samples can be long-term cryopreserved under controlled low-temperature conditions and shared among the CAVD laboratories. GHRC develops novel procedures and helpful technology for optimized sample processing and cryopreservation of clinical specimens collected by CAVD and for the storage of relevant reagents. Key elements of the GHRC technology platform are new cryosubstrates with integrated non-volatile memory chips, the specimen knowledge and preparation workflow management system ChameleonLab, high precision and high throughput controlled-rate freezers integrated in cryoworkbenches, sample access towers shielding cryotanks from ambient air and an electronic cryotank infrastructure allowing accurate sample localization and selective data access even under cryogenic temperatures. To ease collaborative research, a web portal for online registration of HIV specimens has been established enhancing the GHRC central information and specimen administration database. Moreover, GHRC has proved to be well-suited for pseudotype virus stock production and has implemented an appropriate automated large-scale production system.

Keywords: Global HIV Vaccine Research Cryorepository (GHRC); CAVD; cryobank; cryotechnology; cryoelectronics; cryoprocedures; HIV; pseudovirus

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1 Introduction and objectives

The Global HIV Vaccine Research Cryorepository (GHRC), an international consortium coordinated by the Fraunhofer Institute for Biomedical Engineering (Fraunhofer IBMT), is one of the Centralized Service Facilities of the Collaboration for AIDS Vaccine Discovery (CAVD). The CAVD is an international network of scientists and experts dedicated to designing a variety of novel HIV vaccine candidates and advancing the most promising candidates to clinical trials. The network includes fourteen Vaccine Discovery Consortia (VDCs), five Central Service Facilities (CSFs), and two Grand Challenges in Global Health (GCGH) grantees.

The goal of GHRC is to develop one of the most modern global HIV cryobanks for storing and sharing specimens and reagents within CAVD, based on innovating technologies suitable for assuring quality to specimen preparation, to specimen storage and to specimen knowledge management in collaborative scenarios, aiming at comparability and the creation of a reliable HIV-1 related common knowledge base. The GHRC network also develops novel biological procedures for optimized sample processing, cryopreservation and storage of clinical specimens from regional centers, or for reagents generated elsewhere in the CAVD consortium. Also, it provides training and capacity building for regional centers and technology transfer to the CAVD consortium.

Another important project of the consortium is to establish a centralized and standardized HIV-1 pseudovirus production for HIV vaccine trials. The HIV-1 pseudovirus production is a collaborative effort of the Fraunhofer IBMT, the University of the Saarland and the Comprehensive Antibody Vaccine Immune Monitoring Consortium (CA-VIMC) of David Montefiori. In this context, an automated large-scale production system has been implemented to enable GHRC to produce and distribute large amounts of pseudoviruses for HIV-1 vaccine discovery processes.

The entire range of technology developed by GHRC will be made available for HIV/AIDS vaccine development throughout the whole Global HIV/AIDS Vaccine Enterprise. Also the key information about generated reagents as well as the reagents themselves will be available to this network. This will help to ensure that the vaccine candidates will be genetically, immunologically and biologically relevant.

In scope of the GHRC development, a modern and innovative biosafety level 3 (BSL-3) laboratory containing a BSL-3 cryorepository has been established since 2007 at the Fraunhofer IBMT in Sulzbach, Germany. The laboratory operations are performed there according to certified quality control standard (Good Clinical Laboratory Practice, GCLP).

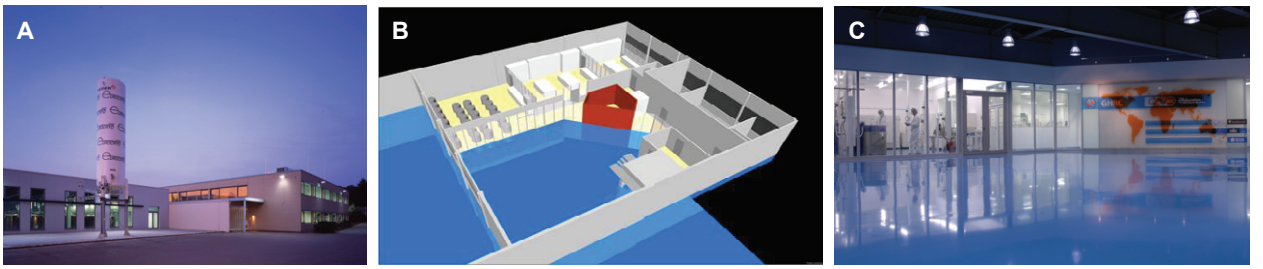


Figure 1: Fully operational HIV Cryobank.

A: Exterior view of the Fraunhofer IBMT Cryorepository with liquid nitrogen tank (25,000l); B: Architect model of BSL-3 laboratory with integrated cryobank; C: completed BSL-3 laboratory with integrated cryobank.

2 Development of an HIV cryotechnology platform

There are some major problems in long-term cryopreservation of biological material. Both intracellular and extracellular ice crystallization lead to cell death, and temperature shifts during transport and sample handling increase the accretion of ice crystals (Fig. 2). In addition, the conventional techniques for sample identification and associating sample knowledge can lead to mistakes and thus, to serious consequences. Especially in collaborative scenarios, quality and comparability of specimen-related knowledge (and thus, the quality of a common knowledge base) are highly dependent on sample preparations and on the accuracy of knowledge acquisition.
Due to those reasons, the GHRC develops techniques, technology and improved procedures for optimized processing, cryopreservation and storage of clinical specimens and for the management of associated knowledge.

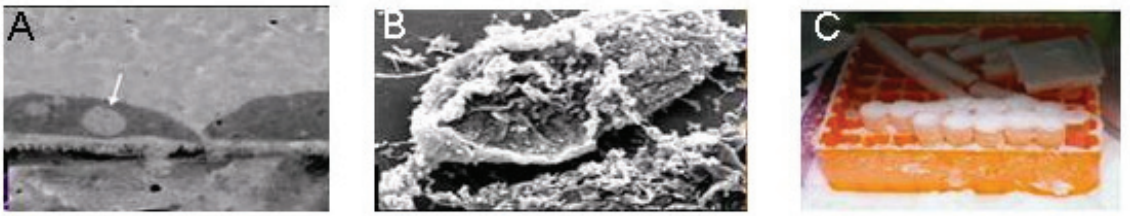


Figure 2: Problems in cryopreservation.
A: intracellular ice crystal; B: destroyed cell by cryopreservation; C: ice formation on surface of cryovials

2.1 Innovative GHRC cryosubstrates and the ChameleonLab

Core of the Fraunhofer IBMT technological platform is the development of discrete cryotubes („cryosubstrates“) with integrated nonvolatile electronic memory chips (Fig. 3). This innovation is motivated by the ambition to achieve the necessary high quality in managing the sample-associated knowledge during long-term cryopreservation, sample transport and sample exchange in collaborative research scenarios. Therefore, the memory chip is intended to provide a reliable sample identification, an unmistakable preservation of existing sample-related knowledge directly on the sample and an easy knowledge distribution. Storing comprehensive sample data directly on the sample, this technique exceeds other concepts for electronical sample identification by far which mainly focuses on, e.g., ID tags referencing sample databases or document management systems. The GHRC quality assurance concerning the knowledge management in cryobanking is unique, providing sample data wherever the sample is located, even under cryogenic conditions. The following figure 3 shows the GHRC cryosubstrates and additional adapters allowing our technology platform to cope even with standard tubes:

Figure 3: Left: Schematic layout of a GHRC cryosubstrate with integrated flash memory chip for decentral knowledge preservation. The substrate consists of the following major sections: lid with inner thread and silicon seal, (B) actual sample compartment (2 ml volume) and

injection-molded 2D- barcoded serial number and (C) socket with integrated additional RFID tag for contactless identification, guide lug and flash memory chip. Right: The currently available cryosubstrates, adapter for standard cryotubes, and sockets with integrated memory chip.

To verify the biocompatibility of our innovative cryosubstrates, we freeze PBMCs in our GHRC cryomedium I (see also section 3 for details). After thawing, we analyze recovery and viability of PBMCs and lymphocytes. Recovery of PBMCs after cryopreservation was high (80-90%), independent of cryotube and volume (data not shown). Also viability of PBMCs frozen in GHRC cryosubstrates compared to cells frozen in standard tubes achieved very good results (97-98%). It is noticeable that the new GHRC cryosubstrates enable an increased PBMC viability (Fig. 4) as well as a reduced amount of dead lymphocytes (Fig. 5) especially for small sample volumes (0.1ml).

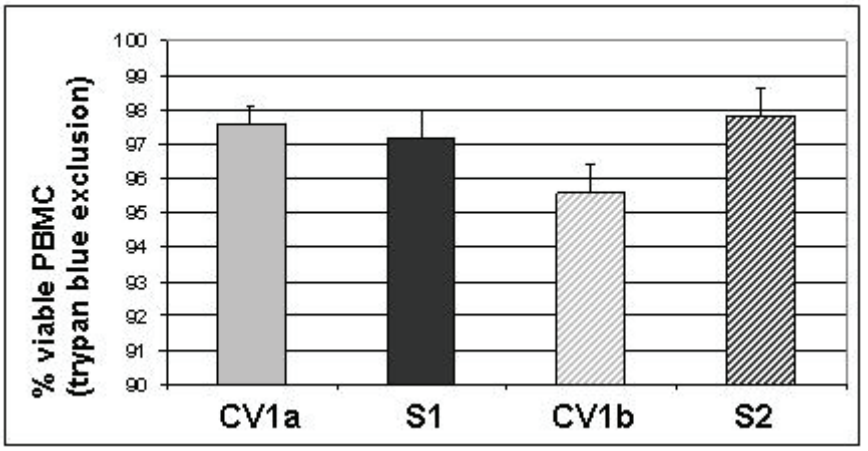


Figure 4: Determination of PBMC viability using trypan blue exclusion: GHRC cryosubstrate vs. standard cryovial
CV1a: 1.8 ml standard cryovial, (filling volume 1 ml), CV1b: 1.8 ml standard cryovial, (filling volume 0.1 ml), S1: 2 ml GHRC cryosubstrate (filling volume 1 ml), S2: 0.1 ml GHRC cryosubstrate (filling volume 0.1 ml).

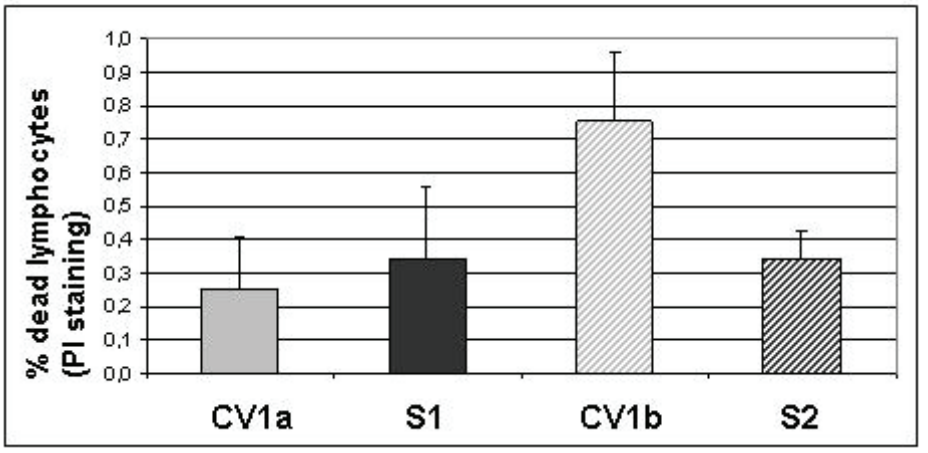


Figure 5: Determination of lymphocyte lethality using FACS analysis: GHRC cryosubstate vs. standard cryovial
CV1a: 1.8ml standard cryovial, (filling volume 1ml), CV1b: 1.8ml standard cryovial, (filling volume 0.1ml), S1: 2ml GHRC cryosubstrate (filling volume 1ml), S2: 0.1ml GHRC cryosubstrate (filling volume 0.1ml).

Concerning collaborative research scenarios, not only the quality of knowledge preservation and exchange is relevant. In contrast, quality of the knowledge itself is highly important whenever comparative studies are used to build a common knowledge base, e.g., about immune responses of PBMCs to potential vaccine candidates. Among organizational data, sample knowledge is mainly created during sample preparation and analysis, and its quality is influenced by a variety of factors, e.g., the performed preparation or analysis steps, the accuracy, detailedness and date of knowledge acquisition (lab documentation), its transcription, e.g., to a document management system, and by the sample quality itself. For generating comparable sample knowledge in collaborative scenarios like GHRC, the comparability of the underlying sample functionality is crucial. Thus, standardized sample preparation and well-frounded, uniform and detailed knowledge acquisition during sample preparation in the laboratory is necessary to allow correlations between biological effects and acquired sample knowledge. Standardizing sample preparation means to minimize the influence of individual, procedural knowledge by providing detailed explicit information for preparing samples of a certain type, but also to avoid mistaking sample and associated preparation protocol. For these purposes and for assuring the quality of sample and knowledge, the staff-supporting ChameleonLab workflow management system has been developed. It is based on the following main principles:

- The sample carries along its complete associated preparation protocol, written in a workflow definition language which can be processed by a workflow engine, in order to enable the sample to ‚control' its own preparation. For this purpose, the workflow definition is stored on the electronic memory chips integrated in the GHRC cryosubstrates and on the common memory chips of carrier plates for standard tubes or storage boxes, together with an initial sample aliquot list.
- The workflow definition is processed by a set of different ChameleonStations in the laboratory, each of them providing a special set of laboratory devices or manual equipment. The ChameleonStations interface the laboratory staff via a GUI (Fig. 6), and supplying detailed instructins on performing each preparation step. They also control available devices through type-specific meta-workflow activities being translated by a device integration framework into the command set of the particular device. This allows easy exchange of workflows between different laboratories.
- The ChameleonStations acquire the generated knowledge from the user through predefined input fields, via a device interface and by automatic time stamp recording for each sample and aliquot. It tracks the creation, preparation success or failure and adjustment of aliquot amounts for each workflow step. The documentation and workflow state is stored in the electronic memory chip of each individual cryotube or sample carrier. This allows easy handling of a running workflow instance and continuing at another ChameleonStation in the same or in a collaborating laboratory, or at a later point of time, e.g., even after transport or after cryopreservation.

Together, the GHRC cryosubstrates and the ChameleonLab allow decentral storage and long-term preservation of all knowledge types identified in biobanking (declarative sample knowledge, procedural sample knowledge and declarative sample knowledge generated by utilizing procedural knowledge) on the sample, and thus its preservation and easy distribution.

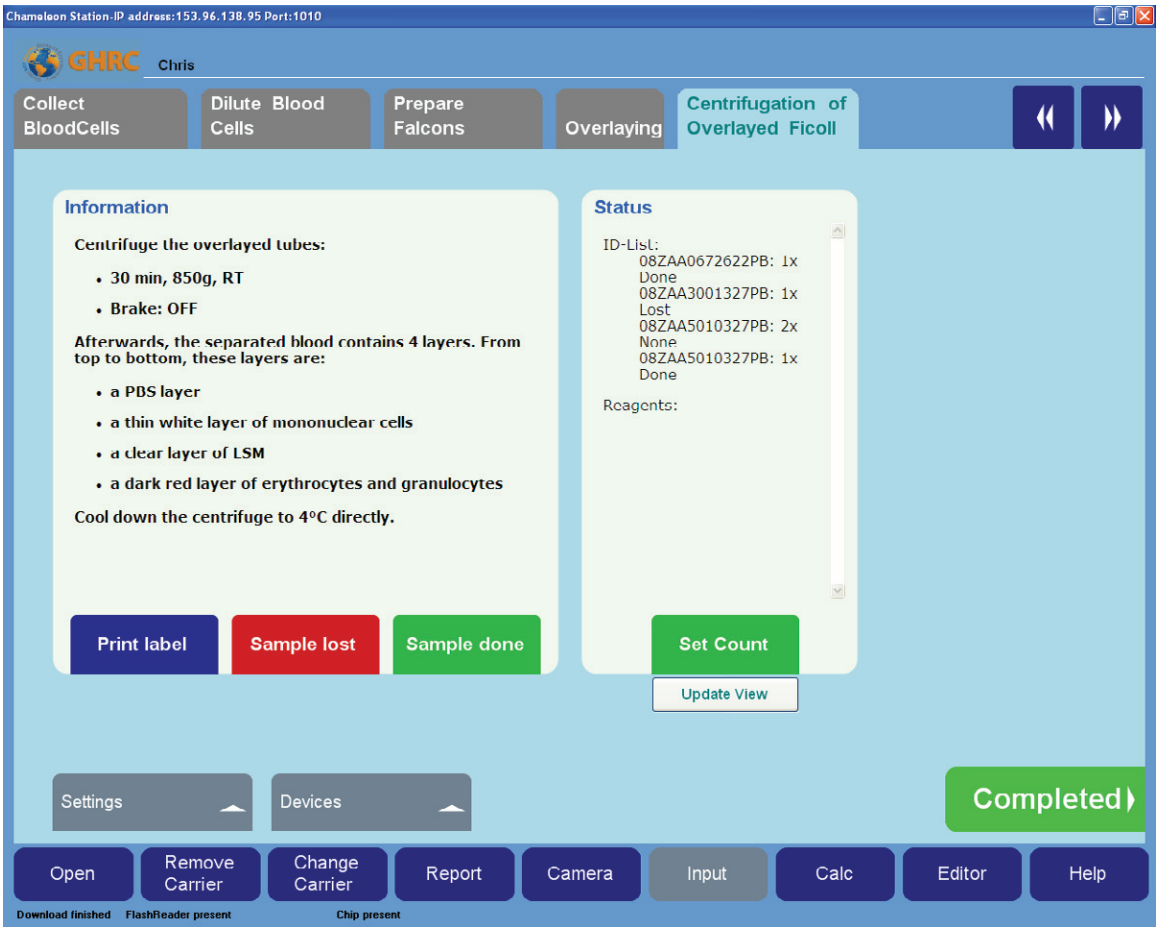


Figure 6: ChameleonStation GUI. Each preparation step is displayed with information area (left) and status area (mid) on an own register tab. This allows thumbing through the whole protocol and preview of the next workflow activity, enabling the user to choose an appropriate ChameleonStation. At the lower area, there are buttons for reading and writing carrier plates / cryosubstrates, turning the camera function of the attached barcode scanner or for generating a printable report on and off. Moreover, general mathematical formulas for calculating, e.g., cell concentration can be accessed by its own button. For ergonomic purposes, the predefined documentation fields can be accessed by the ‚Editor' button whenever manual data acquisition is required from the user. Each preparation step is finished by pressing its ‚Completed' button. Printing labels on demand, registering a sample as ‚done' or ‚lost' and changing the number of its aliquots are further key features of the system.

The whole decentral sample knowledge is organized in a specially designed, flexible and extensible data structure which is also used for controlling the electronic infrastructure of GHRC cryotanks. The tank infrastructure allows the localization of each sample and selected access to its data even under cryogenic conditions during long-term storage. The ‚intelligent' tank infrastructure replaces the conventional ‚dumb' storage racks including drawers. Mechanically, it consists of four quadrant racks. Each of them equipped with a PCB board (‚backplane') with microcontroller and RAM, administrating all of its storage levels (‚wings') and holding an electronic inventory. The wings are implemented as PCB boards communicating with the common backplane of the rack through a serial interface. They interface the individual samples and cryoboxes through a demultiplexer circuit and track their occupied positions. By using a modular architecture, the tank infrastructure is fully scalable and extendable by prospective additional hierachical layers. The single racks are lifted up by the sample access tower shown in section 2.2 which is set on top of a cryotank which contains the intelligent infrastructure. The following figure 7 shows a quadrant rack and its components:

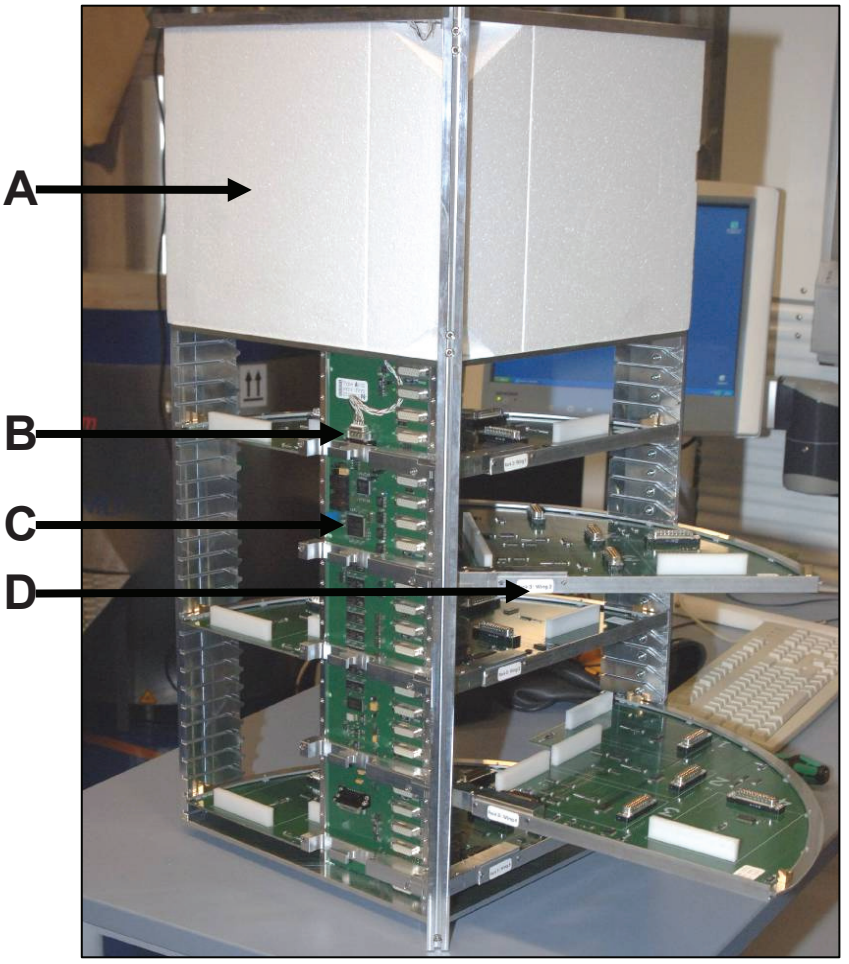


Figure 7: A rack of the electronic tank infrastructure, and its components. (A) thermal insulation at the top of the rack; (B) electronic connection to the tank head electronic; (C) backplane, equipped with microcontroller and inventory memories; (D) modular storage PCBs (‚wings'). The shown rack is modularly equipped with five wings, each of them carrying its own temperature sensors for extending the samples' temperature log during cryopreservation.

2.2 The protective hood concept and its applications

Surrounded by ambient air, conventional sample tube extraction from cryotanks causes both ice formation on surfaces (cryovials, container, tank interior) by air humidity and an interruption of the sample cooling chain. Due to recrystallization, the latter might lead to sample injury and cell death and therefore, to a significant loss of sample quality.

To avoid these problems, GHRC has invented the protective hood concept. It is based on the property of liquid nitrogen evaporating into a multiple of its volume. This effect is used to create a pure nitrogen gas atmosphere artificially within a closed hood shielding, e.g., the tank infrastructure from ambient air and providing temperatures below –80°C. This protective hood concept has been the fundament for the development of a storage tank sample access tower and for the development of a cryoworkbench for sample handling. The clear and dry nitrogen atmosphere allows the handling of samples without ice formation on the surfaces and prevents the electronic infrastructure components from corrosion. A reservoir of liquid nitrogen provides permanent

cooling and gas production. A PID controller monitors all important parameters during operation. The following figure 8 shows model and prototype (3rd generation) of the storage tank sample access tower, being compatible with the quadrant rack system shown in section 2.1 and providing a lift system for accessing the different wings.

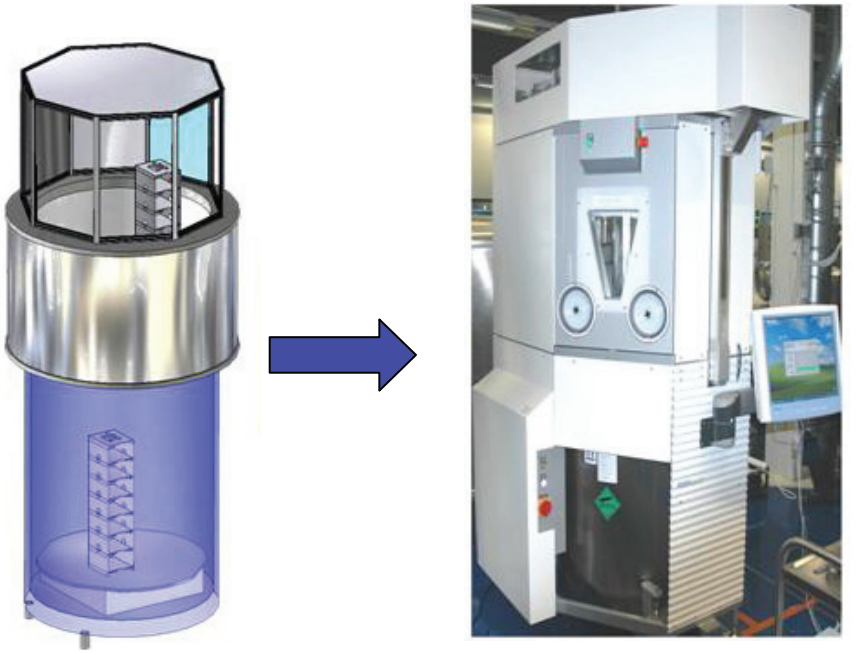


Figure 8: Innovative sample access tower, installed on a sample storage cryotank. The access tower provides an artificially created nitrogen gas atmosphere shielding the infrastructure and samples from ambient air. Moreover, it provides continuous cooling and prevents interruptions of the cooling chain. First prototypes (left) have been constructed by GHRC, the current prototypes (right) for industrial productions are developed in collaboration with Askion GmbH (Gera, Germany).

As mentioned above, the protective hood concept has also been used to develop a cryoworkbench for shielding sample tubes from ambient air while handling. This helps to avoid quality loss by uncontrolled warming and to prevent ice formation on the tubes and their labels. Thus, cell samples can be handled at any appropriate cold temperature. The workbench can be extended with controlled rate high throughput or high precision freezers. The high throughput freezers achieve the needed temperatures by adjusting their vertical position using the temperature gradient within the freezing box. The optimized temperature control of the high precision freezer is achieved by a vaporized nitrogen stream going through an isolated hose and passing a heater. The thermoregulation of vaporized nitrogen instead of liquid nitrogen allows a much better control of the sample chamber compared to the standard market freezer systems. The steady nitrogen steam is responsible for a uniform temperature distribution within the sample chamber. All parameters and important states are visualized and stored electronically. The combination of Access Tower and cryoworkbench with integrated freezers secures the cooling chain for cryosamples and is a major improvement for the cryobanking process quality. We regard both as basic devices for future cryolaboratories.

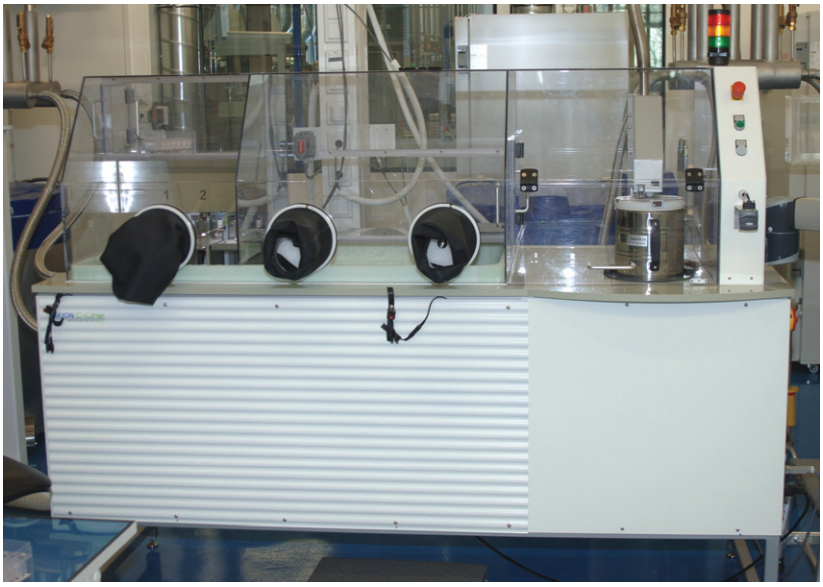
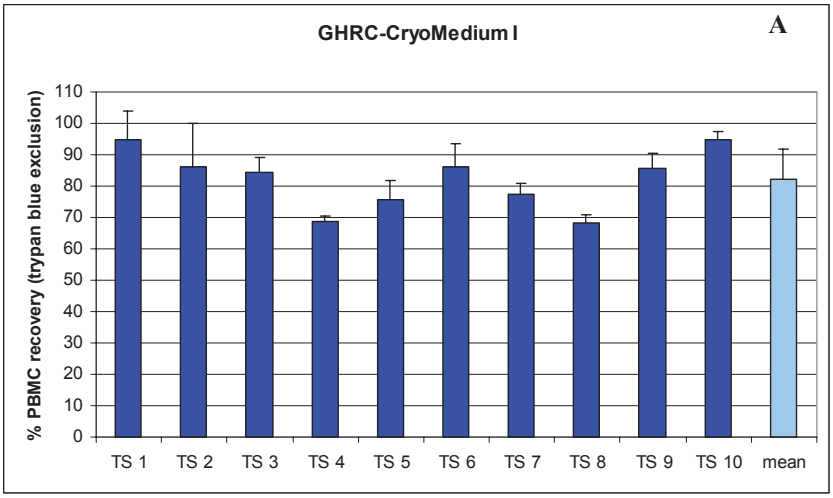


Figure 9: Cryoworkbench with three integrated high throughput freezers (left), sample handling room (middle) and one high precision freezer (right). The shown prototype has been produced in collaboration with Askion GmbH (Gera, Germany).

3 Research on specific cryoprocedures

The successful cryopreservation of living cells requires using a suitable cryoprotectant. Moreover, different cell types must be frozen accordingly to highly specified temperature profiles since the cooling process of a biological sample is determined by physical cell properties such as heat capacity, shape, and size. Cell samples are extremely sensitive to induced changes of salt concentrations in the surrounding medium. The most widely used cryopreservation procedure for cell cultures is based on adding between 5 and 15 % dimethyl sulfoxide (DMSO). However, the amount of DMSO added must be reduced significantly due to its toxic impact on cells at room temperature. This is one major aspect of optimizing cryopreservation.
In this context, the GHRC has also focused on developing methods for successful and efficient cryopreservation of PBMCs which ensure a high survival rate without affecting proliferation characteristics, cell activity or immunological functionality. Those aspects are crucial for a later research use of cryopreserved PBMCs. Therefore, we have developed novel freezing and thawing approaches aiming at the use of cryoprotectants or their combinations having minimum cytotoxicity and maintaining maximum cell functionality. To measure the resulting viability, we have used trypan blue exclusion and FACS analysis as read-out systems by staining with propidium iodide. To measure cell function, we have also used enzyme-linked immunospot (ELIspot) assays. We found out that we can reduce the usual DMSO concentration by addition of hydroxyethyle starch (HES) (6%) to 5% DMSO. We also tested different protein additives in order to substitute the widely used fetal calf serum (FCS) as cryoprotectant and found out that bovine serum albumin (BSA) fraction V is an appropriate additive to substitute the potential mitogenic and/or immune modulating FCS. Based on our experimental results, we could finally design two different, optimized cryomedia. Both contain a reduced amount of the permeant DMSO and the non-permeant (HES) and are standardized serum-free and manufactured under GMP conditions. Using our cryomedia, the mean PBMC recovery rate is more than 80% (Fig. 10) and the mean PBMC viability rate is more than 97% (Fig. 11). Also the cell functionality measured by interferon-γ release in ELIspot assays is optimal with the new GHRC cryomedia I + II (Fig. 12).



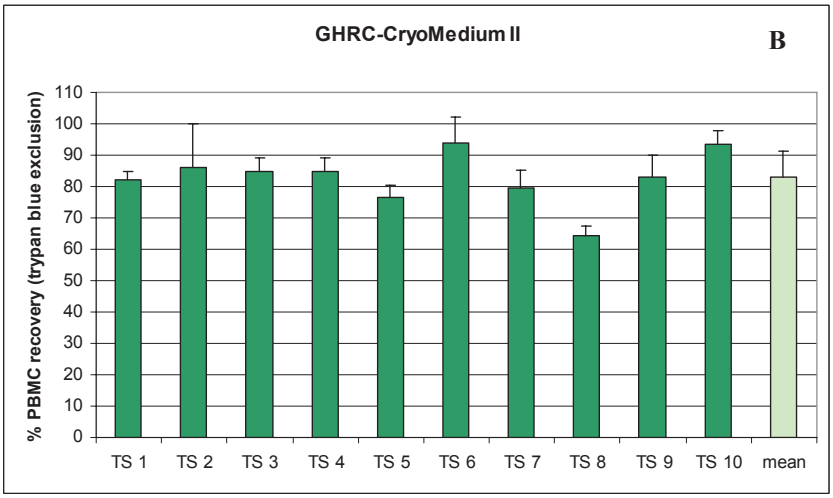


Figure 10: PBMC recovery rate with the GHRC cryomedia I (A) + II (B).
PBMCs from ten different donors had been frozen with a concentration of 10 million PBMCs/ml. After thawing, the PBMC recovery rate was calculated using trypan blue exclusion.

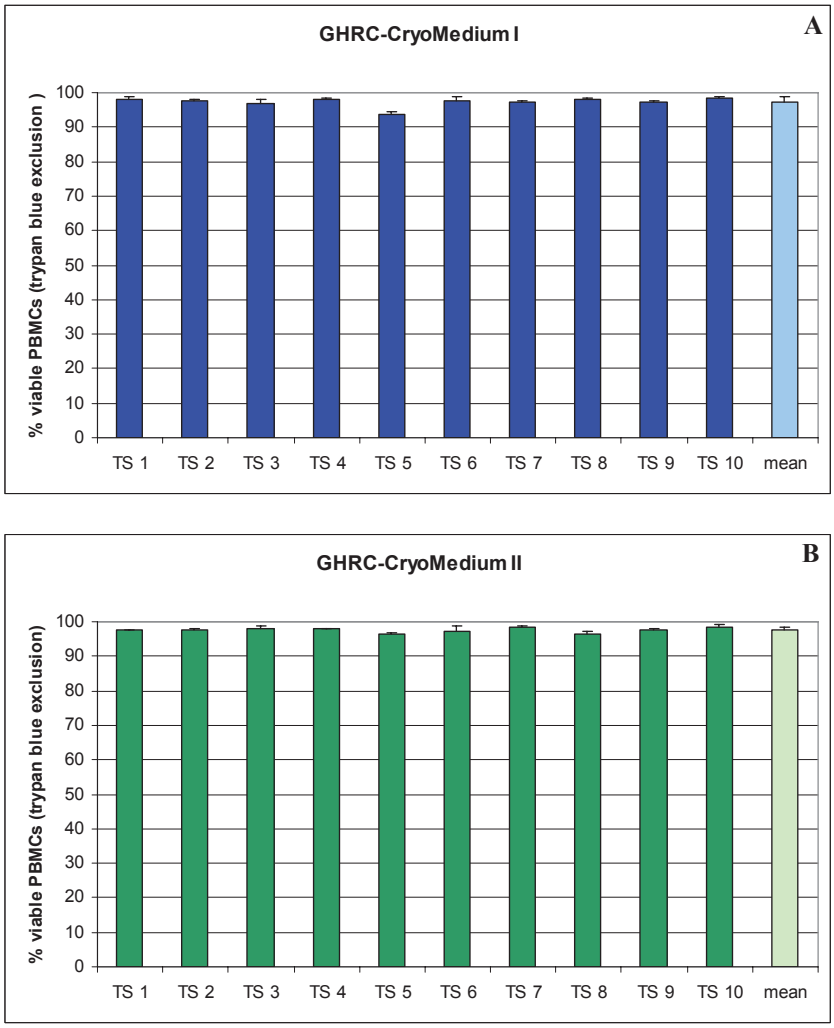


Figure 11: PBMC viability rate with the GHRC cryomedia I (A) + II (B).

PBMCs from ten different donors had been frozen with a concentration of 10 million PBMCs/ml. After thawing, the PBMC viability rate was calculated using trypan blue exclusion.

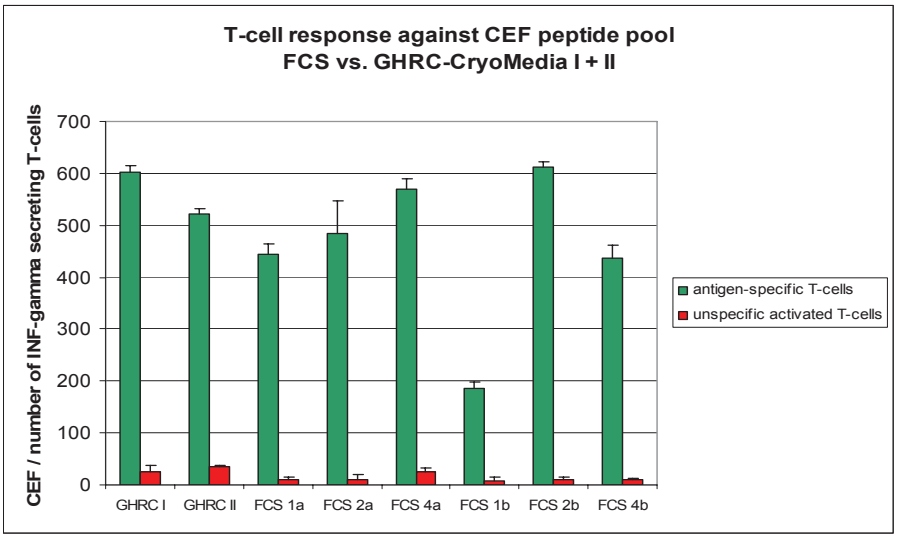


Figure 12: ELISpot analysis FCS vs. GHRC cryomedia. Fetal calf serum (FCS) containing Cryomedia (different batches) has an influence on immunresponse measured by IFN-gamma ELISpot. GHRC cryomedia I + II fully maintain the T-cell functionality.
GHRC I: GHRC cryomedium I; GHRC II: GHRC cryomedium II; FCS 1a: 90% FCS (PAA gold standard), 10% DMSO, FCS 2a: 90% FCS (PAA EU standard), 10% DMSO, FCS 4a: 90% FCS (Invitrogen), 10% DMSO FCS 1b: 70% FCS (PAA gold standard), 10% DMSO, RPMI, FCS 2b: 70% FCS (PAA EU standard), 10% DMSO, RPMI, FCS 4b: 70% FCS (Invitrogen), 10% DMSO, RPMI.

4 Standardized HIV-1 pseudovirus production under GCLP

Standardized assessments of the neutralizing antibody response are a critical component of the HIV-1 vaccine discovery process. Results of the first and second rounds of the developing proficiency testing program for the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells identified the need for the central bulk preparation of molecularly cloned HIV-1 Env-pseudotyped viruses. The GHRC was identified as an appropriate site to produce the large amounts of HIV-1 env-pseudotyped viruses needed by CA-VIMC Core, by regional laboratories and by the broader CAVD community for assessing neutralizing antibody responses in the standardized TZM-bl Cell Assay in support of pre-clinical and clinical studies. A CA-VIMC/GHRC pilot study for pseudovirus production and distribution was conducted April-July 2008. Test data from this study showed that pseudoviruses prepared at the GHRC are equivalent to stocks prepared by the CA-VIMC Central Reference Laboratory (CRL) and other CA-VIMC core labs (Fig. 13). The pseudovirus distribution process also proved the ability of GHRC to facilitate pseudovirus and reagent flow within the CA-VIMC, GHRC and CAVD.
In addition, the GHRC installed the Tecan “Celerity”, a complete cell cultivation and transfection system for automated pseudovirus production in November 2008. The system allows a standardized large-scale production in a closed system which is independent of differences caused by performing the process manually (Fig. 14).

Lab 2

	IC50 µg/ml					ID50 (DIL) HIV (+) Plasma				
HIV pseudovirus	**sCD4**	**4 E10**	**2F5**	**2G12**	**HIVIG**	**1642**	**1679**	**1684**	**BB87**	**BB107**
1) DU156.12	>25 >25	0.39 0.16	>25 >25	>25 >25	336 330	196 249	75 98	187 146	1258 1079	1168 857
2) ZM197M.PB7	14.5 11.9	0.35 0.1	>25 17	>25 >25	374 293	244 380	100 137	184 169	90 129	77 92
3) CAP210.2.00.E8	5.5 4.3	4.6 2.6	>25 >25	>25 >25	855 565	23 45	<20 37	34 96	143 226	<20 <20
	IC80 µg/ml					ID80 (DIL) HIV (+) Plasma				
	sCD4	**4 E10**	**2F5**	**2G12**	**HIVIG**	**1642**	**1679**	**1684**	**BB87**	**BB107**
1) DU156.12	>25 >25	1.3 0.9	>25 >25	>25 >25	1553 1404	35 44	<20 <20	<20 <20	332 268	369 242
2) ZM197M.PB7	>25 >25	2.1 1.5	>25 >25	>25 >25	>2500 2091	34 43	<20 <20	<20 <20	<20 <20	<20 <20
3) CAP210.2.00.E8	17.4 16	19.1 16.6	>25 >25	>25 >25	>2500 >2500	<20 <20	<20 <20	<20 <20	<20 30	<20 <20

IC50 / IC80 for CA-VIMC pseudovirus and HSC pseudovirus deviates < 2 fold

IC50 / IC80 for CA-VIMC pseudovirus and HSC pseudovirus deviates < 3 fold

IC50 / IC80 for CA-VIMC pseudovirus and HSC pseudovirus deviates > 3 fold

Figure 13: Results of neutralization assays with CA-VIMC/GHRC pseudoviruses.
HIV-1 pseudoviruses were successfully produced, QC checked and distributed under GCLP (good clinical laboratory practice). Proficiency tests of 6 testing labs worldwide have been highly congruent.

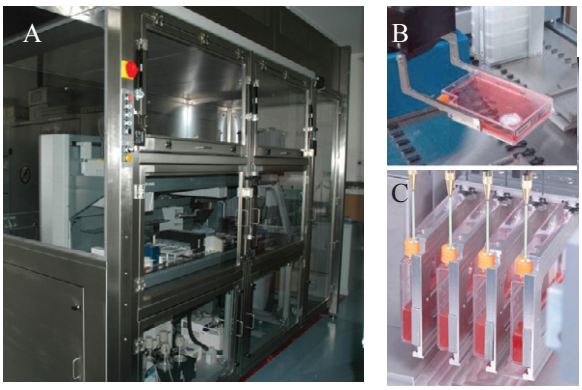


Figure 14: Automation of HIV-1 pseudovirus production. A: The complete closed BSL-2 cell cultivation and transfection system; B: roboflask in robotic manipulator arm; C: roboflask in flipper

5 Conclusions

The GHRC has developed an innovative technological platform for optimized sample storage and knowledge management, suitable for the needs of collaborative scenarios. Moreover, GHRC has established a state-of-the-art BSL-3 specimen repository where HIV-1 related samples are stored both for long-term preservation and for sharing among collaborating laboratories. Optimized cryomedia have been designed. In addition, the centralized and standardized pseudovirus production provides a precious and valuable tool for HIV-1 vaccine trials. Technology, procedures, pseudoviruses and the designed reagents are to be made available throughout the entire CAVD.

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